



PCT/GB 00 / 03450	
REC'D 12	2000
WIPO	PCT INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

GB 00/03450

4

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

Andrew Gersey

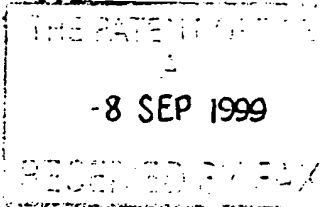
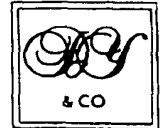
Dated

22 September 2000

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

Patents Form 1/77

Pat 1977
(Ru)The
Patent
Office08SEP99 E475146-1 C03046
P01/7700 0.00 - 9921155.9

Request for a grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference	P007615GB ATM		
2. Patent application number (The Patent Office will fill in this part)	08 SEP 1999		9921155.9
3. Full name, address and postcode of the or of each applicant (underline all surnames)	Medical Research Council 20 Park Crescent London W1N 4AL United Kingdom		
Patents ADP number (if you know it) If the applicant is a corporate body, give the country/state of its incorporation			
	United Kingdom		
4. Title of the invention	Selection System		
5. Name of your agent (if you have one)	D YOUNG & CO		
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	21 NEW FETTER LANE LONDON EC4A 1DA		
Patents ADP number (if you have one)	59006		
6. If you are declaring priority from one or more earlier patent applications, give the country and date of filing of the or each of these earlier applications and (if you know it) the or each application number	Country	Priority application number (if you know it)	Date of filing (day/month/year)
7. If this application is divided or otherwise derived from an earlier UK application, give the number and filing date of the earlier application	Number of earlier application	Date of filing (day/month/year)	

590007001

8. Is a statement of inventorship and ☒ to grant of a patent required in support of this request? Answer 'Yes' if:
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))
- YES

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form 0

Description 10

Claims(s) 4

Abstract 0

Drawing(s) 2

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

11. I/We request the grant of a patent on the basis of this application.

Signature

Date

D. Young & Co.

08 09 99

D YOUNG & CO
Agents for the Applicants

12. Name and daytime telephone number of the person to contact in the United Kingdom Dr A Maschio 023 8063 4816

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

- a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 01645 500505
- b) Write your answers in capital letters using black ink or you may type them
- c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- d) If you answered 'Yes' Patents Form 7/77 will need to be filed.
- e) Once you have filled in the form you must remember to sign and date it.
- f) For details of the fee and ways to pay please contact the Patent Office.

Selection System

The present invention relates to a screening system useful for screening repertoires of DNA binding domains. In particular the invention relates to a screening system based on transcriptional activators of bacterial $\sigma 54$ -dependent promoters.

The majority of proteins involved in cellular functions do so by interacting with other proteins or nucleic acid sequences within the cell. Several approaches have been described that allow an in vivo selection of relevant clones expressing a particular protein or DNA-binding activity. Arguably the most powerful approach are the yeast one- and two hybrid system (Fields & Song 89) for the screening of protein-DNA, respectively protein-protein interactions. However, the two-hybrid system requires an eucaryotic host and consequently the diversity that can be screened is limited. Furthermore the system notoriously suffers from an abundance of false positives.

Larger molecular repertoires can be prepared in bacterial hosts and a number of bacterial systems for the screening of protein-protein and protein-DNA interactions have also been reported. Two systems have been put forward in which the polypeptide chain of an enzyme is expressed in two parts fused to two proteins X and Y and in which cognate X-Y interaction reconstitutes function of the enzyme (Karimova 98, Pelletier 98).

Several in vivo screens for DNA-binding proteins have also been reported (reviewed in Mossing 91, Elledge 89). Each of these methods involves the blockage of a hybrid $\sigma 70$ promotor by the DNA binding protein. Repression of the promotor either prevents the production of conditionally toxic gene or alleviates repression of an antibiotic gene by transcriptional interference. The transcriptional interference assay (Elledge 89) has been used successfully in one case to select DNA binding proteins with altered specificity (Sera & Schultz 96).

Transcriptional activators of $\sigma 54$ dependent promoters have been called bacterial enhancers because their mechanism of activation is superficially similar to the activation of transcription by enhancer proteins in eucaryotes (Kustu 91). Unlike $\sigma 70$ promoters at which the RNA polymerase is bound in an active form and is largely controlled by repression, the $\sigma 54$ RNA polymerase holoenzyme is transcriptionally incompetent. Its conversion into an active form is catalysed by the enhancer protein coupled to hydrolysis of ATP. This unusual mechanism accounts for the low level of background transcription and the enormous difference (10^4 - 10^5) between on and off states in the strongest $\sigma 54$

promoters effected by a single factor. By comparison activators of $\sigma 70$ promoters such as CAP or λ CI increase transcription levels usually by less than 10-fold.

We provide herein a novel screening system which is based on transcriptional activators of $\sigma 54$ -based promoters.

Preferred embodiments of the invention are described in the appended claims.

The Nif family of bacterial enhancers regulate expression of nitrogenase components from $\sigma 54$ promoters in nitrogen-fixing bacteria, and are inhibited by NifL (Austin 94). In bacteria lacking NifL, NifA is constitutionally active. NifA is modular in architecture and we found that this allows for the swapping of the natural DNA-binding domain (DBD) for extraneous DBDs. Such NifA-DBD chimaeras are inactive on the wild type promoter, but activate transcription from hybrid-promoters bearing their cognate target sequences. We propose to prepare and select libraries of NifA-hybrids together with libraries of hybrid promoters. "Crossing" of hybrid libraries will use combinatorial infection, which has been employed successfully to generate very large antibody libraries (Griffith et al 94).

"Generic" libraries are preferably based on a known DBD architectures (e.g. basic leucine zipper, bZIP) and may be derived using PCR amplification with "family-specific" primers. Such libraries may be crossed with hybrid-promoters bearing defined target sequences or libraries of target sequences. In addition to providing information on the distribution of members of the family in a given genome, such libraries may be used to identify and study proteins or molecular compounds that modify DNA interaction within a family of DBDs, for example Tax (from HTLV-1) in the case of bZIP proteins.

In an alternative embodiment, they may also be used to select DBDs which conditionally bind their target sequence only in the presence of other factors such as protein cofactors or small molecular compounds, for example drugs that intercalate into DNA or alter the degree of supercoiling. The system can also be used "in reverse" i.e. to select proteins or molecular compounds that disrupt a particular DNA-protein interaction or to select DBDs that do not bind a particular target sequence or library thereof.

More advanced libraries are preferably derived directly from genomic DNA or cDNA libraries and selected on hybrid promoters bearing a repertoire of target sequences, comprising either a stretch of randomized sequence or a library of inserts derived from fragmented genomic DNA. Data obtained in this way allows the compilation of a genomic directory of DBDs and the building of a promoter-DBD-interaction map.

The NifA chimera also offer the opportunity to better understand aspects of the process of transcriptional activation at $\sigma 54$ promoters. It has been known for some time that binding of the target sequence together with ATP binding promotes oligomerization of NifA. It is believed that it is the oligomer which contacts the polymerase and catalyses the ATP-driven isomerization of the polymerase holoenzyme. Taking advantage of the superactivation effect described above it may be possible to address questions such as which components of the oligomer (e.g. the DNA-bound NifA vs. NifAAC), which are contacting the polymerase and/or coupling ATP hydrolysis to transcriptional activation etc. Furthermore, usage of NifAAC cofactors from different species (possibly together with their diversification by PCR shuffling) may allow identification of the sequence regions critical for transcriptional activation and may allow a "maturation" of the NifAAC coactivator. Indeed, we found the NifA from *K. pneumoniae* to be a superior cofactor to *A. vinelandii* NifA. Finally, it may be possible to use chimera of a known DBD (e.g. GCN4) and a cDNA library as a procaryotic "enhancer" trap, to isolate $\sigma 54$ activators on a genome-wide scale.

Protein-protein interactions may also be selected using the NifA system. Two hybrid proteins are generated: a bait-hybrid comprising protein X fused to a DNA-binding domain and a prey-hybrid comprising protein Y fused to the NifA activation and sensor domains with cognate interaction of X and Y reconstituting the transcriptional activator. The NifA bacterial two-hybrid system could be used for the generation of interaction matrices between cDNA libraries. Ultimately such interaction matrices may yield an interaction map of the proteins of an organism, and may provide an alternative to the yeast two hybrid system.

Systems based on $\sigma 54$ have a number of advantages over the other systems that are available, e.g. the conceptually similar yeast one-hybrid system. A bacterial host allows substantially larger repertoires to be obtained and thus a much larger molecular diversity to be screened. In particular, using combinatorial infection, our system should allow the "crossing" of both NifA-chimera repertoires with libraries of hybrid reporter constructs, thus permitting coevolution of DBD and recognition sites, or coselection of DBD and target sites from genomic libraries.

Because selection in the $\sigma 54$ -based system is based on a positive readout, i.e. activation of transcription it should be less prone to false positives than other approaches relying on the inhibitory effect of the expressed DBD like the transcription interference assay (Elledge 89). In vivo selection in general may result in the selection of novel DBD that are more

attuned to working under realistic conditions, including supercoiling of the recognition site, presence of a large excess of chromosomal DNA and high protein concentration. Another advantage of our system may be the act that extremely low level of expression appears to be sufficient to affect maximum activation of transcription. This may be particularly helpful in the case of DBDs that are prone to aggregation.

The $\sigma 54$ -based systems of the present invention may be further adapted to take into account potential disadvantages of bacterial expression. For instance, *E. coli* expression may be suboptimal for large eucaryotic transcription factors. However, large eucaryotic proteins can usually be split into smaller domains which retain function and are usually readily expressed in *E. coli*.

Examples

NifA from *A. vinelandii* is a well-studied member of the family of bacterial enhancers and it is a positive regulator of the expression of nitrogenase components in diazotrophs. It is inhibited by *NifL* in response to the presence of oxygen or ammonia. When expressed in *E. coli*, which lacks endogenous *NifL* or an equivalent, *NifA* is constitutionally active. Because of the highly conserved nature of the activation mechanism of $\sigma 54$ RNA polymerase, *NifA* is a very strong activator of transcription in *E. coli*.

Like other members of the family of bacterial enhancer proteins, *NifA* is modular in architecture, both structurally and functionally, comprising 3 domains, a N-terminal sensor domain, a central activation domain (AD), and a C-terminal DNA binding domain (DBD). The central activation domain (AD) can activate transcription independent of DNA binding if overexpressed. Thus the DBD's function appears to be primarily to increase the Activator domain's concentration in the promotor proximity.

We have exploited the modularity of the enhancer structure and swapped the natural *NifA* DNA binding domain (DBD) for extraneous DBDs and libraries thereof. Here we describe the activity of these *NifA*-chimeras in the activation of transcription from the $\sigma 54$ dependent promotor *nifH* and hybrids thereof.

Materials & Methods

Media & Reagents

2xTY, MacConkey agar are described elsewhere (Miller 72). Antibiotics were used at the following concentrations: Ampicilline 0.1 mg/ml, Chloramphenicol 10 μ g/ml,

Streptomycin 25µg/ml. Min-lac medium was essentially M9 medium (Sambrook) supplemented with 1mM MgSO₄, 20µM CaCl₂, 2% (w/v) lactose, 2mg/ml casamino acids, 40µg/ml L-tryptophan, 5µg/ml thiamine and appropriate antibiotics. Min-lacX plates were essentially M9 plates supplemented 2% lactose, appropriate antibiotics and 40µg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside).

Strains

TG1ΔK was derived from TG1 (Gibson 94) using the genome integration strategy of (Haldimann 96). Briefly, NifA(*K. pneumoniae*) residues 1-462 was amplified using Pfu polymerase (Stratagene) and primers 1, 2, cut with NdeI and BamHI and cloned into the genome targeting suicide vector pSK50D-uidA2 (Haldimann 96) and transformed into the Pir⁺ host strain BW23473 (Metcalf 96). Vectors were isolated and transformed into the Pir⁻ strain TG1 harbouring the plasmid pINT-ts (Hasan 94). Chromosomal integration was induced by a temperature shift to 42°C, which leads to expression of λ integrase from pINT-ts and simultaneously stops its replication. Integrants were identified by Kanamycin resistance and screened for Nif coactivation. Once obtained TG1ΔK was grown routinely without antibiotic selection.

Constructs

Chimera constructs were based on pDB737 (Austin 95) encoding NifA (*A. vinelandii*) under the control of the T7 promotor in the plasmid pT7-7 (Tabor & Richardson 85). Expression was by leakiness of the T7 promotor. Chimeras were constructed taking advantage of a unique BanII cutting site, in the linker region between the central domain of NifA and the DBD. GCN4 was amplified using Pfu polymerase (Stratagene) and primers 3, 4, 5 and 6, cut with BanII and Hind3 and ligated into pDB737 cut with BanII and Hind3. ERDBD was amplified using primers 7, 8 and 9, cut with BanII and BamHI and ligated into pDB737 cut with BanII and BamHI. Myn was amplified using primers 10 and 11, cut with SalI and Hind3 and ligated into pDB737 cut with SalI and Hind3. The vector p737S1 is derived from pDB737 by replacing the bla gene with aadA conferring streptomycin resistance as the insertion of a fl phage origin for packaging of the vector into filamentous phage particles. Briefly, aadA, was amplified using primers 12, 13 and cut with AatII and ScaI and ligated with pDB737 cut with AatII and DraIII. The resulting vector p737S was cut with AatII, ClaI. flori was amplified using primers 14, 15 and cloned into the cut p737S to give p737S1. NifA-X chimera were transferred from pDB737 to p737S1 by digestion with NdeI, Hind3 (BamHI for NifA-ERDBD).

Reporter constructs were derived from pACYC184 and the vector pMB1 (Buck 86). Briefly the lac-operon (lacZYA) was amplified with primers 16, 17 and cut with BamHI, MscI. the nifH promoter segment from pMB1 was amplified with primers 18, 19 and cut with Hind3, BamHI and the 2 fragments simultaneously ligated with pACYC184 cut with Hind3 and BsaAI to give pMB3. *flori* was amplified with primers 20, 21, and cut with NheI and XmnI and ligated into pMB3 cut both NheI, XmnI to give pMB31.

Selection and screening

Cells were cotransformed either by simultaneous or sequential electroporation with an expressor construct and a reporter construct and grown ON with appropriate antibiotic selection at 34°C in M9-lac and plated out. β -gal expression was scored either on MacConkey or Minlac-X-gal indicator plates or by ONPG enzyme assay of selected colonies (see below).

Enzyme assay

ONPG assays were used to measure β -gal activity were essentially as described by (Kolmar 96). Briefly, 20 μ l of an overnight culture is transferred to a microtiter well and 100 μ l of chloroform saturated Z-buffer (100mM NaHPO₄, 1mM KCL, 1mM MgSO₄, 50mM β -mercaptoethanol, pH 7.0 (Miller 72) was added and the optical density at 600nm determined using an ELISA reader. Cells were lysed by addition of 50 μ l Z-buffer with 0.4% (w/v) SDS and incubated at 30°C for 10 min. 50 μ l of Z-buffer with 4mg/ml O-nitrophenyl- β -D-galactopyranoside were added and the optical density at 420nm was recorded automatically every 15s over a period of 60min. Specific β -gal activity was calculated from the V_{max} as in (Miller 72).

Example 1: NifA chimera with extraneous DNA binding domains activate transcription but only from promoters with a cognate recognition site

To investigate in what way transcription activation by NifA was dependent on the NifA DNA binding domain (DBD) and on native *nif* promoter structure, we prepared NifA-chimeras in which NifA DNA binding domain (DBD) had been replaced by extraneous DBDs of diverse structural architecture. Initially we explored DBDs, which like the NifA wt DBD bind to symmetrical DNA recognition sequences such as the basic leucine zipper (bZIP) DBD of the yeast transcription factor GCN4, the Zn-finger domain of the human estrogen receptor DNA binding domain (ERDBD) and the helix-loop-helix (HLH) DBD

from the human transcription factor Max (Myn) and determined their capacity to activate transcription of a lacZ reporter gene in vivo from a hybrid nifH promotor, in which the NifA UAS had been deleted and replaced by recognition sites for the extraneous DBDs.

- 5 In order to simplify comparison of transcription activation by NifA chimeras with activation by wt NifA, all reporter constructs had a single DNA recognition site. The wt nifH promotor UAS contains three bona fide NifA recognition sites. Deletion of the two sites more distal to the promotor however did not appear to reduce transcription activation in our reporter under conditions tested.

10

Transcription activation by NifA-chimeras was specific in that they only activated lacZ expression from hybrid-promoters bearing their cognate recognition sequences but not from control reporter constructs bearing wild type UAS or a non-cognate site. In analogy to wt NifA the presence of two or more recognition sites (in phase, see below) did not increase activation by the Nif-GCN4 chimera.

15

Activity was also dependent on the phasing of the recognition site with respect to the promotor: when the symmetric ATF/CREB recognition site for GCN4 was offset in increments of 1 bp, optimal activity was observed when the ATF/CREB was centred on the same bp as the symmetric wt UAS. Presumably efficient contact with the RNA Pol holoenzyme requires that the activator be bound on the right face of the DNA.

20

Transcription activation by NifA-chimeras appears to preserve fine specificity of isolated DBDs. Wild-type GCN4 binds with equal affinity to the symmetric ATF/CREB site as well as to the pseudo-symmetric AP-1 site. Indeed, the NifA-GCN4 chimera showed identical levels of transcription activation in reporter constructs with either of these sites. A NifA-ERDBD chimera showed strong activity on a reporter with its cognate ERE site but no activity above background levels with reporters bearing the similar GRE recognition site for the closely related glucocorticoid receptor DBD.

25

30

Example 2: Coexpression of NifA with NifA-chimeras boosts specific transcription activation by NifA chimeras in a specific and DNA independent manner

35

The level of transcription activation by the NifA-GCN4 and NifA-ERDBD chimeras was lower (ca. 10%) than for wt NifA. However, near wt levels of activity (80%) were reached when wt NifA was coexpressed within the same cell as a "coactivator".

The coactivation was independent of DNA binding as a NifA variants in which the DBD had been deleted (NifA Δ C) was found to be just as active as wt NifA. On the other coexpression of an isolated NifA central domain (both the DBD as well as the N-terminal sensor domain deleted (NifA Δ NC)) failed to coactivate. NifA derived from different species showed greatly variable efficiencies as coactivators. NifA variants from *K. pneumonia* (NifA Kp, NifA Δ C Kp) being almost three times as effective as NifA, while NifA variants from *Rhizobium* (NifA Rh1, NifA Rh2) were poorly active as coactivators.

10 The coactivator effect was found to enhance only specific transcription activation but not background levels of transcription from promoters with non-cognate recognition sites. We therefore constructed an *E. coli* strain, expressing NifA Δ C Kp (the *K. pneumoniae* NifA with its DBD deleted) from a weak promoter (phoB) from the chromosome (TG1: Δ K).

15 The coactivation effect has analogies in eucaryotic transcription , for example the enhancer Sp1, in which isolated Sp1 activation domains can stimulate transcriptional activation by the DNA binding-form of Sp1, a phenomenon termed "superactivation".

20 **Example 3: Tethering of NifA chimera at the UAS is sufficient for activation, but strong activation requires correct positioning**

We also investigated transcription activation by NifA-chimeras with asymmetrical recognition sites such as the classic Zn-finger Zif268 as well as the DBD from p53.

25 Both NifA-Zif268 and NifA-p53 chimeras activated transcription, but only at low levels (2 - 5-fold above the background). However, when the Zif recognition site was duplicated, to give a symmetric palindromic site transcription activation increased substantially. Non palindromic duplication of the recognition site in tandem did not increase activation.

30 Thus while simple tethering is sufficient for some activation, only bipartite binding appears to give a strong activation. Presumably, tethering only leads to an approximate positioning of the activation domain with respect to the RNA polymerase holoenzyme, thereby reducing the likelihood of a productive interaction.

35

Example 4: Selection of active NifA-chimeras by lac complementation

Using expression of the lac operon (lacZYA) from our reporter construct as the read-out of transcription activation allows the selection of active NifA-chimera on the basis of metabolic complementation of a Δ lac strain, with lactose as the only carbon source. Initially we spiked populations of NifA-ERDBD with NifA-GCN4 at the ratios $1/10^4$, $1/10^6$ in the presence of the GCN4 cognate reporter ATF/CREB-nifH and grew populations overnight in minimal medium supplied with lactose. Pre- and post selection populations were scored by plating on MacConkey-lactose plates as well as by PCR screening. The results are summarised in Table 1. Selection factors of up to 10,000 -fold per round were observed.

In order to determine to what degree the basic region of bZIP proteins is suited as a general framework of DNA recognition we prepared a library of mutant GCN4 bZIP DBDs (2×10^7 mutants) in which 5 key residues of DNA interaction are randomized and selected it against the natural ATF/CREB recognition site. After just one round of selection a majority of colonies contained active NifA-GCN4 mutants as judged by lacZ expression.

References

- Austin S. et al (1994) *J. Bacteriol.* 176, 3460
- Buck M. et al (1986) *Nature* 320, 374
- 5 Elledge S.J. et al (1989) *Proc. Nat. Acad. Sci USA* 86, 3689
- Fields S. & Song O. (1989) *Nature* 340, 245
- Gibson T. J. (1984) *Studies on the Epstein-Barr virus genome*, University of Cambridge
- Griffith et al (1994) *EMBO J.* 13, 3245
- 10 Haldimann A. et al (1996) *Proc. Nat. Acad. Sci USA* 93, 14361
- Hasan N. et al (1994) *Gene* 150, 51
- Karimova G. et al (1998) *Proc. Nat. Acad. Sci USA* 95, 5752
- Kolmar H. et al. (1995) *EMBO J* 14, 3895
- Kustu S. et al (1991) *Trends Biochem Sci* 16, 397
- 15 Metcalf W.W. et al (1994) *Plasmid* 35, 1
- Miller J.H. (1972) *Experiments in molecular genetics*, Cold Spring Harbour, NY
- Mossing M.C., Bowie J.U. & Sauer R.T. (1991) *Methods Enzymol.* 208, 604
- Pelletier J.N. et al (1998) *Proc. Nat. Acad. Sci USA* 95, 12141
- 20 Sambrook J. et al (1990) *Molecular cloning-a laboratory manual*, Cold Spring Harbour, NY
- Sera T. & Schultz P.G. (1996) *Proc. Nat. Acad. Sci USA* 93, 2920
- Tabor S. & Richardson C.C. (1985) *Proc Natl Acad Sci USA* 82, 1074

Claims

- 1) A method for the screening and selection of protein-DNA interactions and protein-protein interactions comprising a chimeric transcriptional activator of $\sigma 54$ promoters.
- 5 2) A method for the screening and selection of protein-DNA interactions and protein-protein interactions comprising a chimeric constitutionally active transcriptional activator of $\sigma 54$ promoters.
- 10 3) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1) comprising a chimeric transcriptional activator of $\sigma 54$ promoters in which activation resulting from cognate interactions is enhanced by the coexpression of a full length or variant form of the $\sigma 54$ transcriptional activator.
- 15 4) A method for the screening and selection of protein-DNA interactions in bacteria comprising a chimeric transcriptional activator comprising a transcription activating sequence and
- 20 a) a DNA binding sequence
b) a domain with DNA binding activity
c) a library of sequences or domains with DNA binding activity
- 25 5) A method for the screening and selection of protein-protein interactions in bacteria comprising a chimeric transcriptional activator comprising a transcription activating sequence and
- 30 a) a protein binding sequence
b) a domain with protein binding activity
c) a library of sequences or domains with protein binding activity
d) a library of random peptides
- and a chimeric DNA binding protein comprising a DNA binding domain and
- a) a protein binding sequence
b) a domain with protein binding activity
c) a library of sequences or domains with protein binding activity
d) a library of random peptides
- 35 6) A method for the screening and selection of protein-DNA interactions in bacteria comprising a chimeric transcriptional activator as in 4) and a hybrid $\sigma 54$ promoter

controlling directly or indirectly the expression of one or more reporter genes conferring a selectable phenotype. The hybrid $\sigma 54$ promotor comprises a $\sigma 54$ binding site, and

- a) a binding site for integration host factor
- b) a binding site for a DNA bending protein
- c) an intrinsically bent DNA sequence

as well as

- a) a recognition sequence for the enhancer (UAS)
- b) a recognition sequence for an extraneous DNA binding protein
- c) a library of recognition sites
- d) a stretch of random DNA sequence

7) A method for the screening and selection of protein-protein interactions in bacteria comprising a chimeric transcriptional activator as in 5) and a hybrid $\sigma 54$ promotor controlling directly or indirectly the expression of a reporter gene conferring a selectable phenotype. The hybrid $\sigma 54$ promotor comprises a $\sigma 54$ binding site, and

- a) a binding site for integration host factor
- b) a binding site for a DNA bending protein
- c) an intrinsically bent DNA sequence

as well as

- a) a recognition sequence for the enhancer (UAS)
- b) a recognition sequence for an extraneous DNA binding protein

8) A method for the screening and selection of protein-DNA interactions and protein-protein interactions in bacteria comprising a chimeric NifA.

9) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1) comprising a chimeric NifA in which activation resulting from cognate interactions is enhanced by the coexpression of a full length or variant form of NifA.

10) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1) comprising a chimeric NifA from *Azotobacter vinelandii* in which activation resulting from cognate interactions is enhanced by the coexpression of a full length or variant form of NifA from *Klebsiella pneumoniae*.

11) A DNA sequence; a vector or a linear segment of DNA comprising a hybrid $\sigma 54$ promoter as in 5) in which the promoter is the *nifH* promoter from *A. vinelandii*. and the reporter genes are

- a) metabolic markers such as the lac operon (*lacZ*, *lacY* and *lacA*)
- b) conferring a fluorescent phenotype, such as GFP
- c) conferring antibiotic resistance such as Zeo
- d) conferring another selectable property

12) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1), 2) and 3) in which selections are carried out in the presence of molecular compounds that modify protein-DNA interactions, for example by altering the structure of the DNA binding protein (e.g. Tax for bZIP proteins) or the DNA structure (e.g. through intercalation or affecting supercoiling) or modify protein-protein interactions (e.g. allosteric effects, creation of new binding surfaces).

13) A method for the screening of target ligands in which selections are carried out in the presence of target ligands that modify protein-DNA and protein-protein interactions.

14) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1), 2) and 3) in which selections are carried out in vivo (in bacteria) or in vitro (in vitro transcription and translation).

15) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1), 2) and 3) in which selections are carried out in *E. coli* comprising a chimeric NifA as in 10) and a hybrid $\sigma 54$ promoter as in 11).

16) A kit for research purposes comprising a chimeric transcriptional activator of $\sigma 54$ promoters and a wild-type or hybrid $\sigma 54$ promoter or libraries thereof.

14

17) A method for the screening and selection of activators of $\sigma 54$ promoters (enhancer trap) comprising hybrid $\sigma 54$ promoter comprising the target site for a known DBD (e.g. GCN4) as well as a chimera of the DBD and

a) a $\sigma 54$ activator domain

5

b) a library a real or putative $\sigma 54$ activator domains

c) a library of sequences or domains

d) a genomic library

10 18) A target ligand identified by method 12) and 13)

19) A DNA binding protein and/or target site identified by method 1) - 10)

15

20) A protein and/or its target ligand identified by method 1) - 10)

21) An activator of $\sigma 54$ promoters identified by method 17)

20

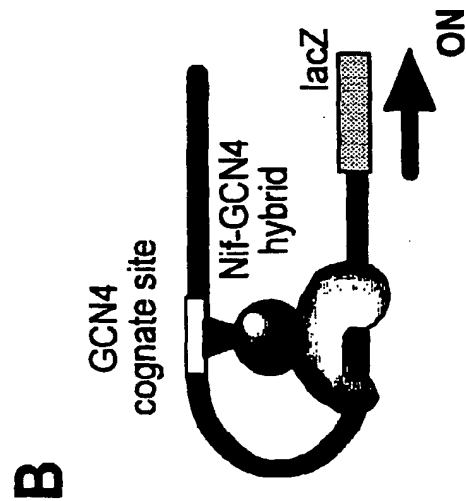
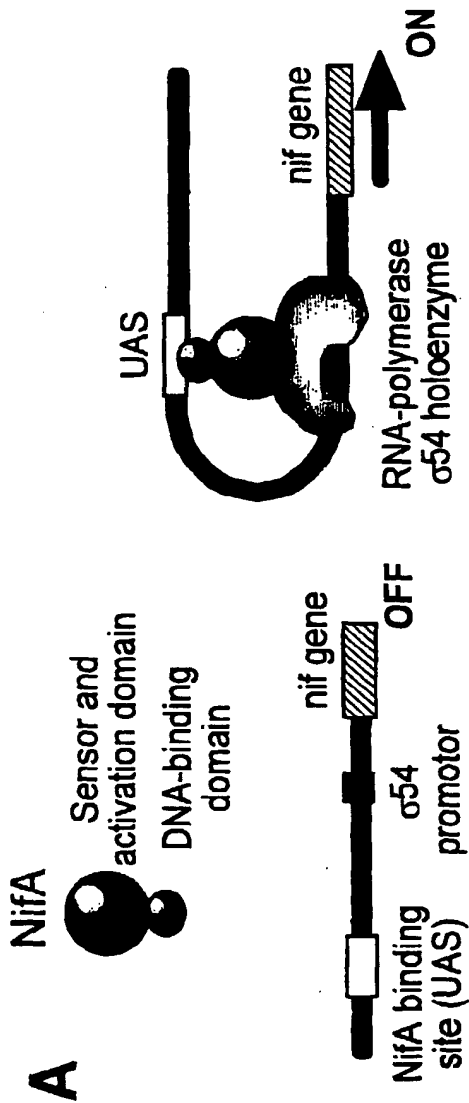
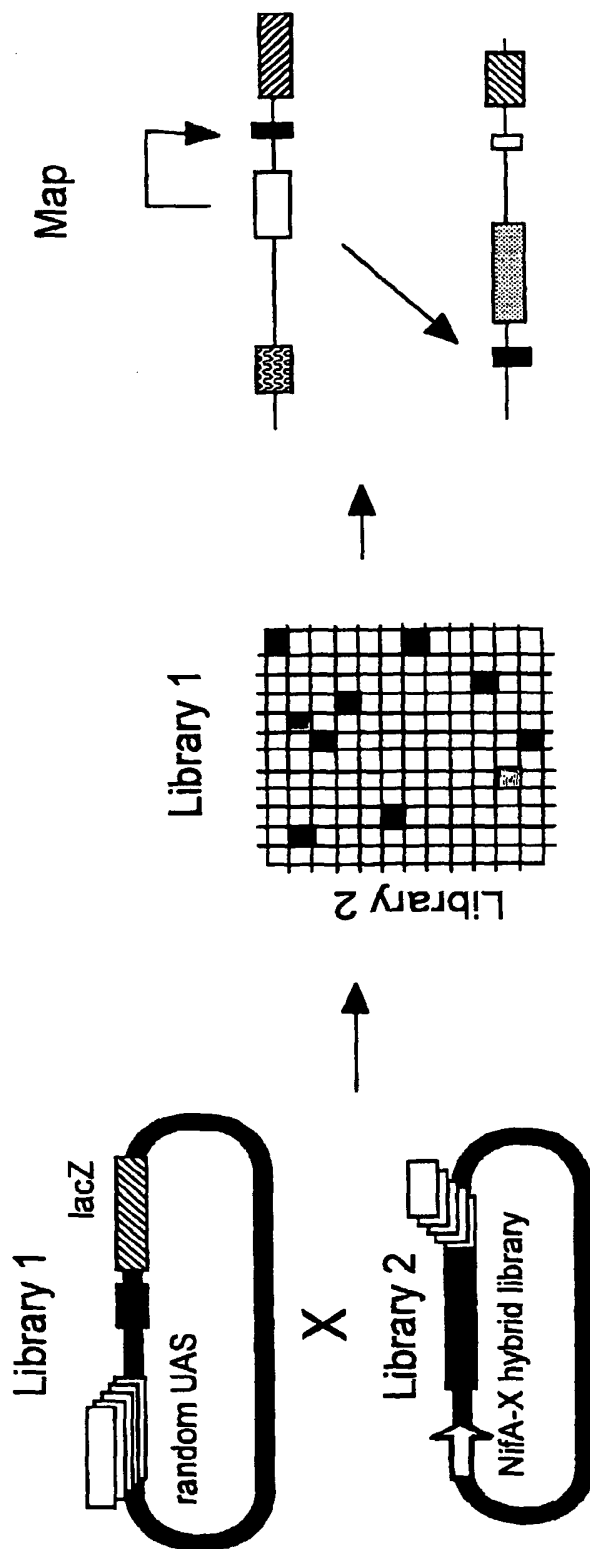




Figure 2



PCT/GB00/03450

D. Young & Co

8/9/00